PAPER NO. 3

APPLICATION OF FLOW CYTOMETRIC METHODS IN IMMUNOTOXICOLOGY

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INTRODUCTION

Flow cytometry is an instrumental technique that permits the simultaneous, quantitative measurement of a variety of properties on individual cells in a flow stream at rates of several thousand cells per minute. In addition, many instruments provide the capability to sort cells on the basis of preselected determinants. Examples of parameters that can presently be measured by commercial instruments include: single or dual wavelength fluorescence, low or 90° angle light scatter, cell number, cell size or volume, and others. Fluorescence provides the basis for the majority of applications and has been utilized in the development of methods for the analysis of DNA cell cycle phase, cell surface marker expression, phagocytosis and viability in suspensions of single cells. These analytical techniques make possible the study of chemically-induced alterations in cell proliferation and differentiation, specific cell functions, such as phagocytosis and cytotoxicity, and the identification of specific cell populations.

METHODS

INSTRUMENTATION

The design of the flow cytometer is similar to that of a fluorometer, the three primary components being a source of excitation (usually a laser), a means of sample containment (flow stream), and a detector(s). A schematic diagram is presented in Figure 1. A suspension of single cells is introduced into a stream by means of a laminar flow chamber adjusted so that the cells pass through in single file. A highly efficient excitation source is required in order to measure fluorescence on individual cells. This is achieved in most instruments using a tunable laser light source, although some instruments employ a mercury arc lamp. Fluorescence detection is accomplished by placing photoelectric detectors perpendicular to the exciting light beam. Specificity is obtained by using a combination of a monochromatic light source and appropriate barrier filters. Tunable argon or krypton lasers are readily available and

provide a variety of useful excitation wavelengths. A feature that discriminates cell sorters from simple cytofluorometers is the ability to separate cells on the basis of predetermined analytical criteria. This is accomplished by vibrating the sample stream at a given frequency to break it into droplets containing individual cells at a measureable distance after analysis has occurred. When the cell satisfies predetermined sort criteria, a charge is placed on the droplet containing that cell which is then deflected either to the left or the right by a pair of deflecting plates.

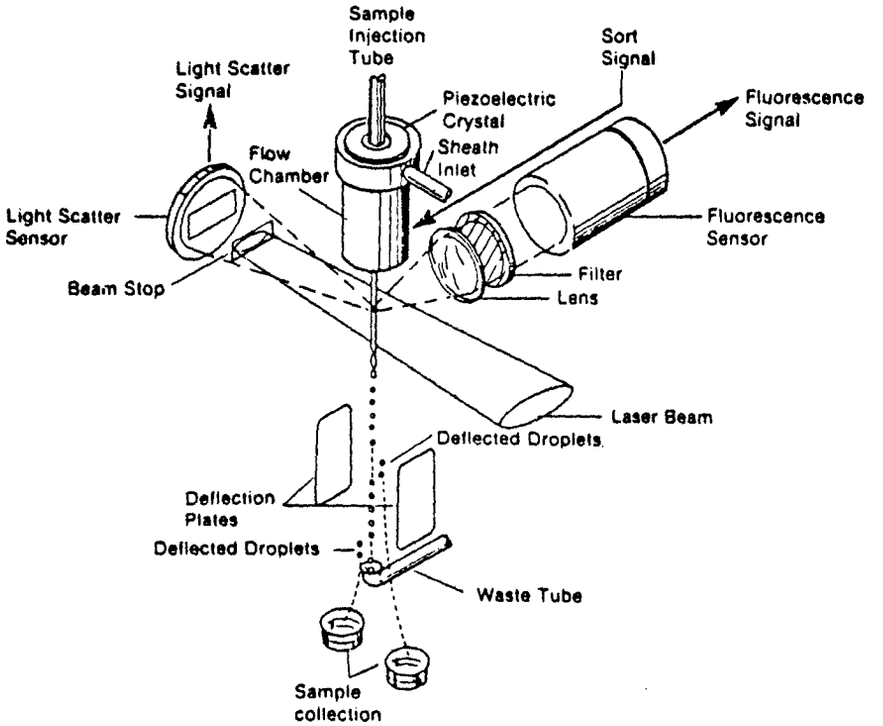


Figure 1. Schematic diagram of a laser based cell sorter (flow cytometer). (Courtesy Coulter Electronics, Hialeah, Florida.)

PARAMETERS

LIGHT SCATTER

When a cell or particle passes through a beam of coherent polarized light (laser beam), light is scattered in all directions. Low angle light scatter or light scattered in the forward direction (0.5-20°) is largely due to diffraction from biologic membranes and under certain circumstances can be used to discriminate cells on the

basis of size. Refractive index also contributes to low angle light scatter, and the measurement is most often used to discriminate between live and dead cells. Right angle light scatter (90°) is largely dependent on internal structures. By measuring low and 90° angle light scatter simultaneously it is possible to distinguish erythrocytes, granulocytes, lymphocytes and macrophages in whole blood. We have used this technique to monitor cell populations in bone marrow as well (Figure 2).

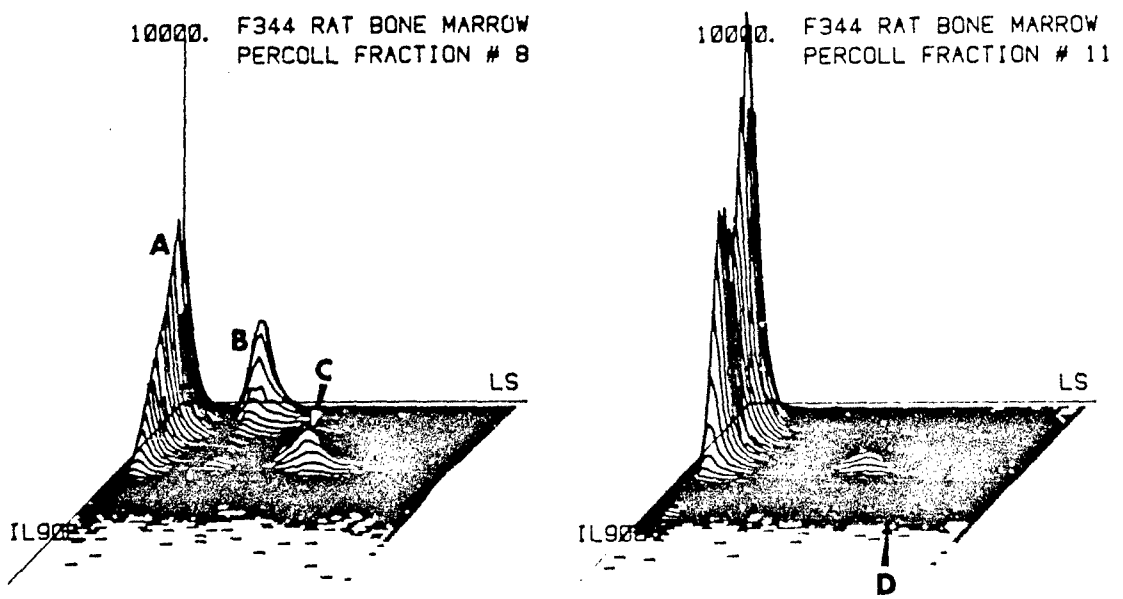


Figure 2. Three dimensional projections of low angle light scatter (LS) versus 90° angle light scatter (IL90) versus cell number for F-344 rat bone marrow cells fractionated on Percoll® (Pharmacia Fine Chemicals) gradients. A - Red Blood Cells; B - Lymphoid Cells; C - Myelocytic and Monocytic Precursor Cells; D - Megakaryocytes and Granulocytes.

FLUORESCENCE

Fluorescence detection methods are predicated on the ability of a fluorescent substance to be taken up and/or bind to specific structures on or within the cell. Analysis of DNA content in individual cells is based on the use of fluorescent compounds that stoichiometrically bind to DNA and for which the intensity of fluorescence is proportional to DNA content. Under these conditions, fluorescence intensity can be used to quantitate the proportion of the total cell population in each phase of the cell cycle. Examples of staining reagents that have been used for this purpose include

the interchelating dyes, ethidium bromide and propidium iodide, and the "groove binding" dyes, mithramycine and chromomycin. Cells must be fixed in order to allow entry of the latter substances as well as fluorescein into the cell, and is the basis for a number of live-dead cell assays.

Immunofluorescence can be used to identify and quantitate cells on the basis of surface markers or membrane antigens. Antibodies directed against surface immunoglobulins or alloantigens can be used to discriminate between cell types (e.g., B and T lymphocytes) and to monitor differentiation in these populations. The use of two fluorochromes, combined with the recent commercial availability of dual laser systems, provides the capability to discriminate cells on the basis of several determinants, simultaneously.

MISCELLANEOUS

A number of additional parameters or techniques have been theoretically possible for some time and have recently become commercially available. These include: time-of-flight, coulter volume, slit scan, and fluorescence anisotropy. In addition to direct measurements of cell size or volume, these techniques allow for analysis of particle shape functions (time-of-flight), cell surface marker distribution (slit scan), and membrane fluidity (fluorescence anisotropy).

Detailed descriptions of cytofluorometric instrumentation and its applications in biology have been presented elsewhere (Horan and Wheeless, 1977; Melamed et al., 1979; Irons, 1981).

APPLICATIONS IN IMMUNOTOXICOLOGY

CELL CYCLE ANALYSIS

The cell cycle can be divided into four phases on the basis of DNA synthesis: G1, the first gap or period of no measureable DNA synthesis; S, the period of active DNA synthesis and replication; followed by G2, the second gap during which DNA synthesis is complete and the DNA content of the cell is twice the diploid content found in G1; and M, or mitosis. Additional compartments, such as Go, can be considered on the basis of RNA or protein synthesis; however, analysis of G1, S, and G2/M phase distributions enables the study of growth kinetics in a given tissue or population of cells. Quantitation of the DNA content of individual cells provides a means to determine in what phase of the DNA cycle they reside. Normal bone marrow and stimulated cells in culture contain cells undergoing asynchronous exponential growth. Asynchrony is defined by two characteristics of growing cells, namely, that they move through the cell cycle independent of one another and that the transit time for any phase is independent of the transit time for any other phase.

Therefore, at any given point in time, the probability that a cycling cell will be in a particular phase of the cell cycle is proportional to the amount of the total cell cycle transit time occupied by that phase. In a theoretical asynchronous population, the distribution of DNA content among cells consists of a single absolute value for G1/Go, a linear continuum of values for DNA content in S phase, and a single value for G2/M that is exactly twice that of G1/Go. An actual DNA distribution histogram is presented in Figure 3A and illustrates the contribution of experimental error, both instrumental and biological. The single values theoretically described for Go/G1 and G2/M are replaced by gaussian curves. Pretreatment of the animal with a cycle specific agent results in a partial synchronization, in this case accompanied by an accumulation of cells in G2/M (Figure 3B). Quantitative compartmental analysis of DNA histograms can become extremely complex, and several computer based methods exist for parametric as well as nonparametric histogram analysis. We have previously used this approach to study the effects of repeated benzene administration on cell cycle kinetics in rabbit and rat bone marrow (Irons et al., 1979; Horan et al., 1980; Muirhead et al., 1980).

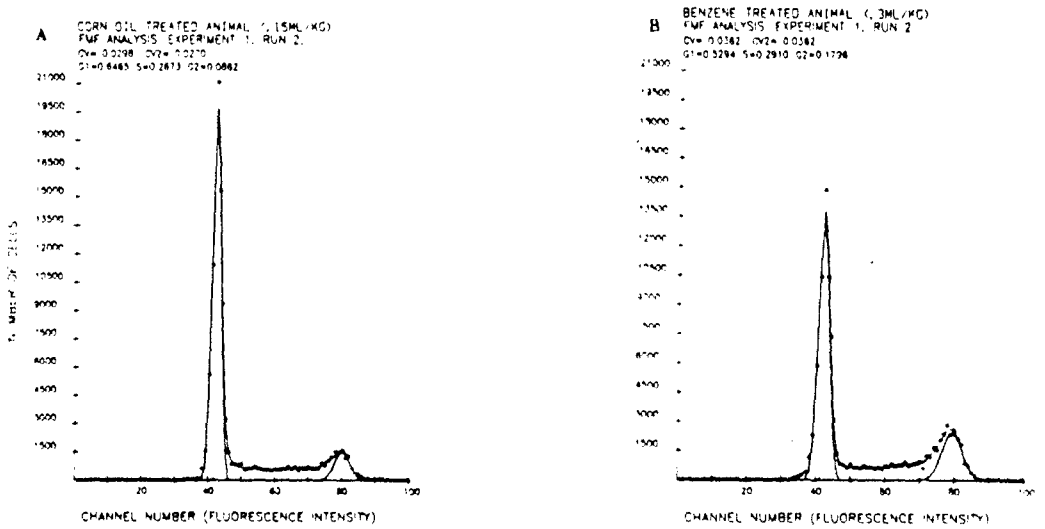


Figure 3. Two dimensional histograms of DNA content in bone marrow cells of (A) control and (B) benzene treated NZW rabbits. Corn oil or benzene in corn oil was administered daily for three days. CV, Coefficient of variation for G1 and G2 peaks, respectively. Values for G1, S, and G2 are the fraction of total cells in each compartment. (Mithramycin stained.)

ANALYSIS OF MACROPHAGE FUNCTION

CYTOSTASIS

A parameter often used to measure macrophage function is target cell cytostasis or cytotoxicity. This is usually monitored by measuring the uptake of ^{3}H-thymidine into tumor target cells or determining the effect of activated macrophage populations on this process. We have recently applied quantitative DNA cell cycle analysis for this purpose and have found it to exceed conventional tracer methodology in both sensitivity and resolution. Murine macrophages were activated by the eliciting agents BCG or maleic anhydride vinyl ether (MVE-2) as previously described (Dean et al., 1978) and resident peritoneal macrophages harvested and cultured with a leukemia cell line (MBL-2). Cytostasis of leukemia target cells, as measured by ^{3}H-thymidine incorporation, was significantly depressed only after 48 hours, whereas cell cycle analysis proved sensitive enough to monitor changes as early as 12 hours. Furthermore, cell cycle analysis revealed that macrophages activated with different eliciting agents altered the kinetics of cytostasis in culture (Table 1).

TABLE 1. ASSESSMENT OF MACROPHAGE FUNCTION USING LEUKEMIA TARGET CELL CYTOSTASIS[1]

Incubation Time (Hr)	Percent of Leukemia Target Cells in S Phase[2]			
	MBL-2 Cells Alone	Macrophages		
		Control	BCG-	MVE-2-Elicited
12	----	21.5	0.0	21.6
18	42.6	29.8	6.5	10.7
24	64.2	37.5	0.0	0.0
48	64.2	69.8	23.0	12.7
	^{3}H-THYMIDINE INCORPORATION (DPM)			
48	38,599	27,673	11,601	15,992

1 Resident peritoneal macrophages were harvested from untreated B6C3F1 mice or mice previously injected with BCG (14 days previous) or MVE-2 (6 days previous) and purified by adherence to plastic microexudate flasks.

2 Based on counts of 20,000 target cells/culture.

PHAGOCYTOSIS

The ability of macrophages to phagocytize fluorescein labeled plastic microspheres is also a measure of macrophage function and can be assessed using cytofluorometric methods (Figure 4). This

technique not only provides a means to enumerate phagocytic cells but also the capability to quantitate phagocytosis on an individual cell basis. This technique can be applied to the assessment of phagocytosis both in vivo as well as in vitro.

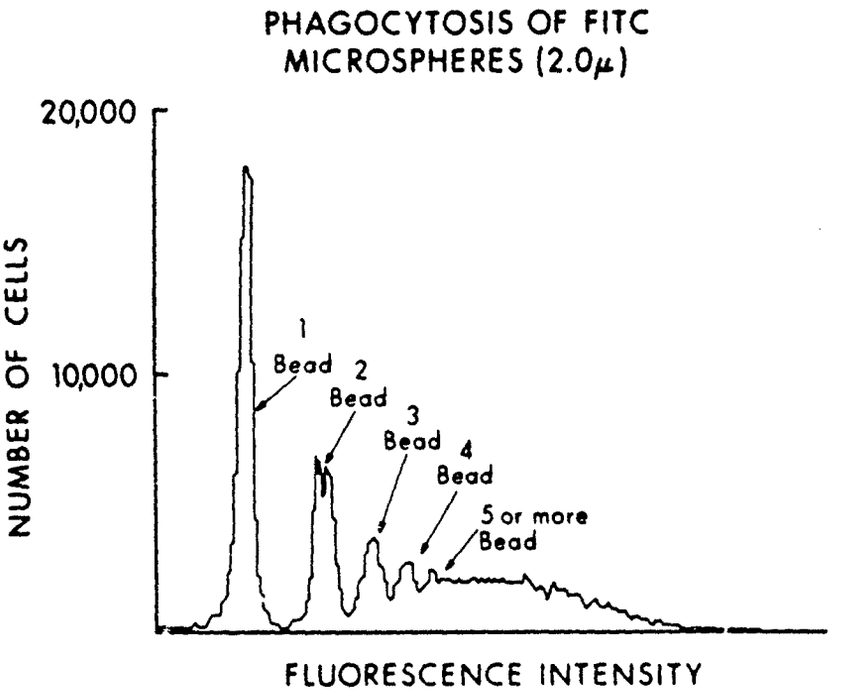
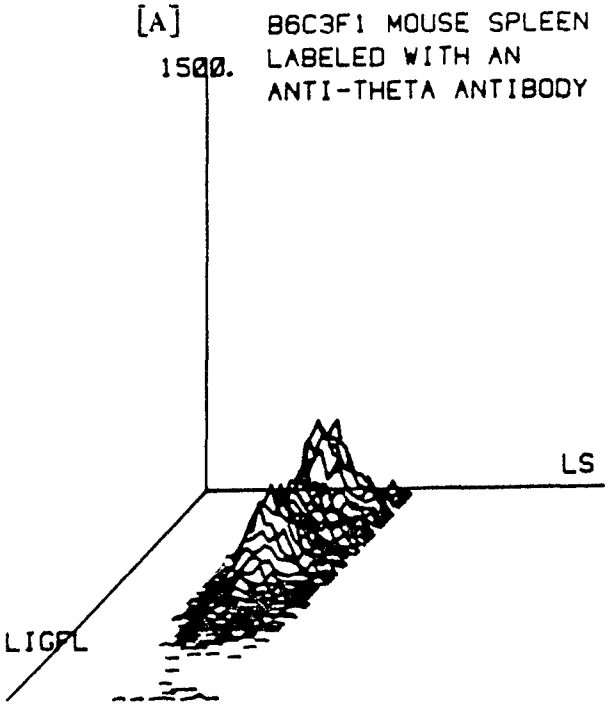


Figure 4. Quantitative cytofluorometric analysis of macrophage phagocytosis using fluorescein isothiocyanite conjugated Covaspheres© (Covalent Technology).

CELL SURFACE MARKER ANALYSIS

Cytofluorometric analysis of cell surface antigens has become an important immunologic technique for the identification of lymphocyte subpopulations and in the study of lymphocyte differentiation. It can be used to quantitate surface marker expression on individual cells as well as enumerate cells bearing particular markers indicative of cell type or level of differentiation. For example, T and B lymphocytes can be enumerated using fluorescent antibodies directed against theta antigen (Figure 5a) or surface immunoglobulin (Figure 5b), respectively. In addition, expression of surface alloantigens accompanying functional differentiation, such as those expressed by T helper or suppressor lymphocyte subpopulations, can be employed to assess lymphocyte differentiation in vivo and in vitro by quantitating the number of cells expressing differentiation markers in culture or in tissue under controlled conditions (Irons et al., 1981).



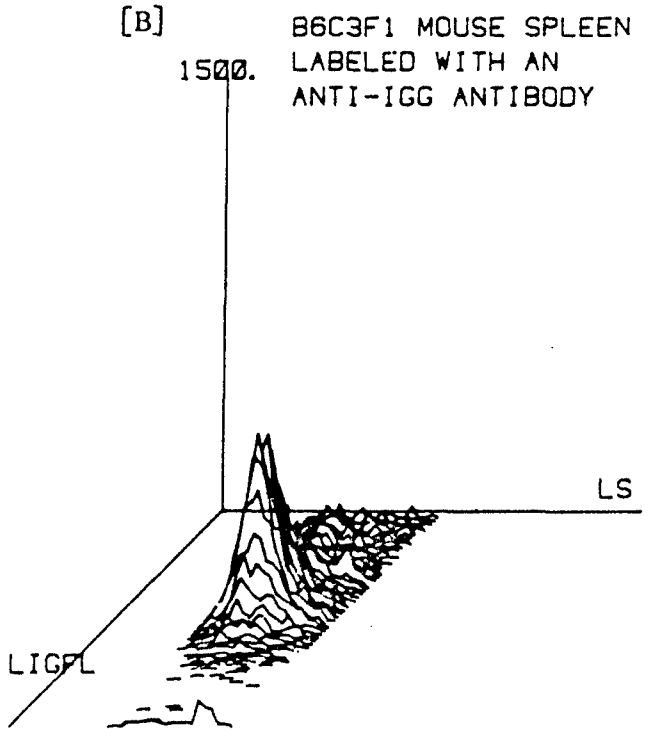


Figure 5. Examples of cytofluorometric analysis of T and B lymphocytes in B6C3F1 mouse spleen.

CONCLUSION

Cytofluorometry provides a sensitive and powerful tool that is being widely exploited in cell biology in general and immunobiology in particular. It offers the advantage of measuring a wide variety of parameters on individual cells. Although flow cytometry has only recently been applied to the field of toxicology, its use is limited only by the imagination of the investigator and should greatly facilitate the evaluation of the effects of chemicals on such phenomena as cell growth, differentiation, and function.

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